VARIOUS METHODS OF DETERMINING THE BACTERICIDAL ACTION OF SUBSTANCES IN VITRO AND THEIR RELATION TO THE CHEMOTHERAPY OF BACTERIAL INFECTIONS *

STUDIES IN PNEUMONIA, II

JOHN A. KOLMER, S. SOLIS-COHEN, AND GEORGE D. HEIST

From the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, Philadelphia

In the rational development of the chemotherapy of bacterial and protozoan infections it is desirable, if possible, to commence experimental work with a substance or substances possessing some definite destructive effect on the microparasite under study. This parasitotropic effect may be apparent only in test-tube experiments; in such event, an effort is made to lower the toxicity or organotropic effect of the substance, with, or even without, an increase of its parasitotropic power, in order that it may be administered in such quantity as will exert in the living animal an inhibitory or killing action on the microparasite under study without injury to the host.

The ultimate aim in bactericidal chemotherapy is the discovery or the rational and systematic development by synthesis, of a substance that is strictly monotropic; that is, one possessing a selective affinity for the protoplasm of a particular microparasite and exerting a specific killing effect on that organism. Thus far this desideratum has not been achieved; as even arsenobenzol (salvarsan) is not strictly parasitotropic for Spirochaeta pallidum, but possesses also a marked parasitropism for other spirochetes and other protozoa, notably various trypanosomes. While the development or discovery of substances which are markedly bacteriotropic or protozootropic in general constitutes an important advance, the ultimate aim, as stated, should be the synthesis of a strictly monotropic substance. It is suggested that further studies with arsenobenzol tending to increase its parasitotropic effect on spirochetes alone and Spirochaeta pallidum in particular, will still further increase the therapeutic efficacy of this drug.

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'LEADS' IN CHEMOTHERAPEUTIC RESEARCH

In the present state of our knowledge of chemotherapy, chance or accidental discovery must play an important rôle in the discovery of a 'lead.' Substances are to be selected or prepared on as systematic a basis as possible, and tried out by actual experiment; those yielding encouraging results are then subjected to various systematic modifications with experimental trial of the new compounds. In this manner chemotherapeutic research proves to be costly and laborious, as amply demonstrated by the prolonged and costly series of experiments directed by Ehrlich, which resulted in the discovery of arsenobenzol.

In the discovery of leads and the study of new compounds, animal experiments are of primary importance, not only because these are the sole means of determining the organotropic or toxic effects of the compounds, but because they are the sole means of determining the actual parasitotropic or therapeutic effects. In conducting these experiments it is necessary to select a protozoon or bacterium that yields a uniform infection of the animals of not too severe a character, and to reproduce as far as possible the same lesions in the animals as are found in man. The test microparasite should either produce definite lesions easy of detection and study or cause the death of the animal in a given period of time. For studies in bacterial chemotherapy virulent cultures of the pneumococcus are admirably adapted to work with mice and rabbits; in studies of protozoa, T. equiperdum or T. brucei is valuable, the white rat being used as host.

These experiments, however, are likely to prove costly, and, as in the case of tuberculosis, it may be many weeks or months before results can be determined. Furthermore, special animals such as monkeys or the higher apes may be necessary for the determination of the parasitotropic effect of a substance, as in the case of anterior poliomyelitis and syphilis, in which the particular microparasites fail entirely to infect such animals as rabbits, guinea-pigs, and rats, or, at least, fail to do so with sufficient uniformity. In chemotherapeutic studies in syphilis the rabbit may be employed, altho the infection usually pursues in this animal a brief course tending to spontaneous recovery. For these reasons an effort should be made to train or adapt a race of the particular microparasite under study to survive and multiply in the tissues of an animal easily obtained and handled, so that the abundance of experimental material needed in chemotherapeutic studies on a large scale may be available. In addition to this, as stated,

an effort should be made as far as possible, to reproduce in the experimental animals lesions similar to those found in man. For example, the effect of a drug in the blood of a rabbit or white mouse in a pneumococcus bacteriemia must be different from that in the exudate of a consolidated lung in pneumonia of man.

In chemotherapeutic studies on syphilis, experience has shown that a trypanosome may be used, as T. equiperdum, or a spirochete other than S. pallidum, as S. gallinarium, to determine the parasitotropic effect of new compounds as they are produced; because those compounds showing marked effects on these microparasites, as for example arsenobenzol, also produce a profound effect on S. pallidum in human and experimental syphilis. It remains to be determined, however, whether similar conditions hold true in the chemotherapy of bacterial infections; that is, whether a compound showing a high bactericidal effect in vitro, or even in vivo, on one microorganism, as B. typhosus or the pneumococcus, will show a similar effect on the microorganisms of other diseases as tuberculosis or anterior poliomyelitis.

THE RÔLE OF BACTERICIDAL TESTS IN VITRO IN BACTERIAL CHEMOTHERAPY

In chemotherapeutic studies on bacterial infections, experiments in vitro may be said to have a positive value in preliminary orientation in the development of leads and the study of new compounds as they are produced. Experimental data at hand tend to show that substances possessing a high bactericidal activity in vitro, and particularly in a menstruum of fresh sterile serum, are more likely to exert an inhibitory example, Morgenroth's drug, optochin (ethylhydrocuprein), and its hydrochlorid exert a very high bactericidal action on the pneumococcus hydrochlorid, exert a very high bactericidal action on the pneumococcus in vitro and are likewise effective to some extent in vivo; other cinchona derivatives, including certain salts of quinin, possessing more or less bactericidal value in vitro are likewise effective to a certain degree in vivo.[1] Arsenobenzol has been found to possess the highest parasitotropic activity on T. equiperdum in vitro of a number of substances tested,[2] and, as well known, this drug exerts the best therapeutic or parasitotropic effects in vivo. Unfortunately, however, other substances that are highly bactericidal in vitro, as the mercurials, also possess a high degree of toxicity for the living animal, and all efforts

[1] Cohen, Kolmer, and Heist, Jour. Infect. Dis., 1917, 20, p. 81.
[2] Kolmer, Schamberg, and Raiziss, ibid., p. 10.

of the chemotherapeutist have so far failed to lower materially the toxicity of these compounds.

Since the time of the earliest discoveries in bacteriology and of bactericides, substances highly bactericidal in the test tube have been known to fail to exert any appreciable influence in vivo when given in safe doses. This lack of effect may be due to the high organotropic or toxic effect of the substance on the body cells in general or on a particular group of cells of vital centers, precluding the use of a bactericidal quantity; to insolubility and difficulty of administration; to rapid union with the proteins of the body and the formation of inert compounds; to rapid elimination; to failure to reach the microorganism in a lesion, and to still other causes. Of these possibilities the first mentioned is of primary importance; but the method and manner of attack of the bactericidal drugs on the protoplasm of cells, aside from coagulation, is almost unknown. Nevertheless, it is possible by systematic modification of the molecule through addition, removal, or substitution of certain atom groups, to produce in some instances a sufficient lowering of toxicity to give an available therapeutic agent. The demonstration of this fact constitutes the great triumph of Ehrlich and opens a fascinating field of research to chemist and biologist.

It is highly probable that experimental studies tending to increase the monotropism of a drug in vitro and particularly in a menstruum of serum will prove of value in chemotherapeutic work. For example, ethylhydrocuprein, which shows the highest selective action upon the pneumococcus in vitro, likewise proves most bactericidal in vivo. Similar facts may be proved in the future in connection with the microparasites of tuberculosis and of typhoid fever, and staphylococci, and other infective bacteria.

On the other hand, a substance failing to exert parasitotropic action either in vitro or in vivo may still prove of value as a basis of composition, and offer a valuable lead in chemotherapeutic research. For example, arsenic in the form of the trioxid has no appreciable effect on T. equiperdum in vivo and but slight effect in vitro,[2] and yet it forms the basis of arsenobenzol, which is so highly parasitotropic both in vitro and in vivo.

Likewise, it is possible that a chemical may be more efficacious in vivo than in vitro by reason of the formation of new and more active compounds in vivo; or by exciting the body cells to produce antibodies for the parasitic antigen or chemically facilitating such production; or by the stimulation of phagocytosis, as suggested by some of our experi-

ments in the case of quinin compounds in pneumococcus infections.[3] Still, as a general rule, it appears that substances without appreciable effect in vitro are likely to be similarly inert in vivo. Arsenobenzol offers no exception to the rule; contrary to the general impression, it possesses a marked trypanocidal activity in vitro.

It is highly probable, therefore, that experiments in vitro have a definite value in chemotherapeutic research as methods of preliminary orientation and in the development of monotropic chemicals. This value is greater when the identical microparasite causing the definite infection under study is employed in the tests. In the absence of a pure culture of the particular microparasite against which a destroyer is sought, or in the presence of insuperable technical difficulties, other and more easily cultivated organisms closely or remotely related, or even of a different biologic order, may be employed; as in the use of B. typhosus and other bacteria by Jacobs and his colleagues[4] in chemotherapeutic studies concerning anterior poliomyelitis.

SPECIAL REQUISITES OF BACTERICIDAL TESTS IN VITRO IN BACTERIAL CHEMOTHERAPY

It is well known that many factors influence the results of bactericidal tests, and many investigators, notably Rideal and Walker,[5] and Anderson and McClintic[6] have sought to establish standard methods for the bacteriologic standardization of disinfectants. Consideration of the physical and chemical laws underlying the action of chemicals on bacteria in vitro are to be found in their papers, and in that of Phelps[7] and others.

For chemotherapeutic studies 2 main kinds of in-vitro tests may be employed: (1) a simple technic observing the first principles governing these reactions, particularly a uniform temperature, and employing an easily cultivated microorganism, as B. typhosus; or (2) a technic aiming at conditions more closely approximating those operative in the living animal.

As a result of a large amount of work with various methods, one fact stands out clear, and that is the necessity of adopting a uniform technic. The most marked variations in bactericidal power appear as the test-methods employed are varied.

[3] Kolmer, Steinfield, and Cohen, Jour. Infect. Dis., 1917, 20, p. 101.
[4] Jour. Exper. Med., 1916, 23, p. 569.
[5] Jour. Roy. San. Inst., 1903, 24, p. 424.
[6] Jour. Infect. Dis., 1911, 8, p. 1.
[7] Ibid., p. 27.

With certain microorganisms, as B. typhosus and staphylococci, either the Hygienic Laboratory, or the Rideal-Walker method may be employed to determine the bactericidal action of new compounds developed in the course of chemotherapeutic studies. But, as we shall point out later, these methods are not adapted to work with microorganisms less hardy and requiring special culture media, as the pneumococcus, gonococcus, and S. pallidum. Furthermore, it has appeared to us that for the special purposes of chemotherapeutic study an acceptable method should approach as nearly as possible the conditions presented in the living animal, particularly with reference to temperature.

Here we may summarize what we consider to be the essentials of an acceptable in-vitro bactericidal test for the special purpose of chemotherapeutic studies in bacterial infections. All these factors have been subjected to experiment, using in most instances quinin compounds and various types of pneumococci.

1. The test should be conducted at a temperature of 37 C. instead of 20 C. in order to approach more nearly conditions in the living animal. The influence of temperature on the chemical reaction between bacterium and bactericide has been uniformly emphasized and constitutes a very important factor.

2. The solution of chemical should contain some organic matter, as the protein of broth, sterile inactivated serum, or ascites fluid. Wide variations in bactericidal power are noted according to whether the menstruum is serum, pus, or salt solution.

According to Wright[8] and his co-workers, Morgenroth's drug has almost the same bactericidal power in vitro in a solution in serum as in salt solution, while the bactericidal activity of other substances is greatly reduced; our own experiments tend to confirm this important observation as to cinchona derivatives in general, and demonstrate the probable value of using serum or ascites fluid in these tests.[9]

When large numbers of new compounds are to be tested at frequent intervals, and particularly in an effort to build up a monobacteriotropic chemical, the simpler technic fulfilling at least the fundamental laws of chemical reaction and utilizing sterile salt solution as the menstruum, may be employed; but when an effort is being made to obtain from in-vitro tests data of greater value in relation to the probable effect of a given parasitotrope in vivo, serum is to be preferred.

3. In the chemotherapeutic study of a definite infection the causative microorganism should be employed whenever possible. For example, ethylhydrocuprein hydrochlorid, quinin salts, and other cinchona derivatives show a highly selective affinity for the protein of the pneumococcus, and in employing in-vitro tests in the chemotherapeutic study of pneumococcus infections, these observations demonstrate the necessity of using the homologous organism.

It would also appear advisable to employ a freshly isolated culture from human or experimental lesions in the conduct of certain tests which aim to

[8] Lancet, 1912, 11, pp. 1633, 1701.
[9] Cohen, Kolmer, and Heist, Jour. Infect. Dis., 1917, 20, p. 40.

be conclusive. This has been found especially true of the pneumococcus. The presence or absence of capsules and the degree of virulence of the strain for white mice and rabbits appear to influence markedly the results of tests in vitro. For routine tests, however, it is better to employ a uniform and known culture or cultures well adapted for growth under artificial conditions, but preferably retaining some degree of virulence.

It is necessary to employ in the tests a sufficient number of microorganisms to yield well-defined results. In work with the pneumococcus and similarly less hardy bacteria, appropriate culture media must be employed and frequent counts made by the plating of 24-hour cultures to determine the approximate numbers of living microorganisms present.

ANTISEPTIC AND BACTERICIDAL TESTS

Tests may be conducted after ordinary methods with low concentrations of the chemical over long periods of time, with apparent inhibition of the microorganism which may amount to true killing (the 'antiseptic' test); or with higher concentrations but shorter periods of exposure, and a definite attempt to determine whether or not killing has resulted (the 'bactericidal' test). Plating methods attempt to measure the degree of bactericidal activity after a definite exposure to varying concentrations of the chemical, according to the number of bacteria killed.

In so far as the application of these tests to conditions in the living animal is concerned, it is probable that the antiseptic test is more closely parallel, since in both, the chemical is highly diluted and the exposure prolonged. Furthermore, the number of microorganisms required for definite results can be much less than in the bactericidal test with higher concentration and shorter exposure. Except for accuracy in study, and in maladies in which time may be of high therapeutic importance, there is no real reason for this differentiation between antiseptic and bactericidal properties; not only because they depend on identical reactions, but also because prophylactically and therapeutically a slow rate of killing over a long time may be just as efficacious as a rapid rate in a shorter time.

As pointed out by Phelps[7] and amply confirmed by our work,[9] antiseptic power, or true disinfection at low concentrations for long periods, will show a relation between two chemicals quite different from that shown at higher concentrations and shorter periods of exposure. One compound may be a superior bactericide in high concentration and lose rapidly in efficacy with dilution and hence prove

to be a poor antiseptic; while the other, tho of lesser value in high concentration, retains its power better with dilution.

OBJECT OF THIS INVESTIGATION

With these considerations in mind, we have devised or modified a number of different methods with the definite purpose of determining their advantages, disadvantages, and relative values as bactericidal tests in chemotherapeutic studies on bacterial infections, particularly in pneumonia. The results of these tests with a large number of quinin compounds and other substances acting on pneumococci belonging to the serologic Types I, II, and III, are given in a previous paper.[9]

RIDEAL-WALKER AND HYGIENIC LABORATORY METHODS AND MODIFICATIONS OF THESE FOR BACTERICIDAL TESTS WITH PNEUMONIA

While these methods have proved of distinct value in testing the bactericidal value of chemicals against such microorganisms as B. typhosus and staphylococci, they have proved, in our experiments, unsuited for work with the pneumococcus. With the addition of 0.1 c.c. of a 24-hour dextrose-broth culture of a pneumococcus to 5 c.c. of varying dilutions of the bactericide in distilled water at 20 C., subculturing with a 4-mm. loop of platinum wire nearly always resulted in sterile controls, or at least gave very irregular results. For this reason we have modified these methods with the object of obtaining uniform results and adapting them to work with the pneumococcus in bacterial chemotherapy, (*a*) by conducting the tests at 37 C. instead of 20 C.; (*b*) by having present some organic matter in the solutions of chemical; (*c*) by using larger amounts of culture in the tests; and (*d*) by employing selective culture media for the seed tubes.

Media.—In all our experiments we have employed the broth found by Cole and his associates to be well adapted for cultivating the pneumococcus, namely, one of fresh beef containing 0.1% dextrose, with an end reaction of + 0.2, and sterilized for a minimal length of time. Two drops of sterile, freshly defibrinated human or rabbit blood were added to each tube and all incubated for at least 48 hours to ensure sterility before use. Ten cubic centimeters of the broth were placed in each tube. As these broths vary in their ability to support the pneumococcus cultures, it was found advisable to test out each lot before accepting it for work.

Organism.—Twenty-four-hour broth cultures of stock pneumococci belonging to the first three serologic types were used; but for routine work Type I was finally selected. All cultures were first examined for purity by stained smears and the supernatant cultures used without shaking or filtering, as smears generally showed the cocci to be well scattered. One cubic centimeter

TABLE 1

RESULTS OF BACTERICIDAL TESTS BY MODIFIED RIDEAL-WALKER AND HYGIENIC LABORATORY METHODS

Name: hydroquinin hydrochlorid (methylhydrocuprein).
Culture used: pneumococcus, Type II, 24-hour broth culture; 0.0001 to 0.0002 c.c. fatal for white mice in 48 hours.
Temperature of medication: 37 C.
Proportion of culture and chemical: 1 c.c. + 5 c.c.
Organic matter present: protein constituents of the broth.
Subculture media: special blood broth containing 0.1% dextrose.
Reaction: + 0.2.
Quantity in each tube: 10 c.c.

Substance	Dilution	Exposure in Minutes						Phenol Coefficient	
		2½	5	7½	10	12½	15		
Phenol	1:200	—	—	—	—	—	—		
	1:300	+	—	—	—	—	—	200 |8000	40.0
	1:400	+	+	—	+	—	—		
	1:600	+	+	+	—	+	+	400 |24000	60
	1:800	+	+	+	+	+	+		2 |100
	1:1200	+	+	+	+	+	+		50 =
Hydroquinin	1:8000	—	—	—	—	—	—		
	1:12,000	+	—	—	+	—	—		Coefficient
	1:18,000	+	+	—	—	+	—		
	1:24,000	+	+	+	+	+	—		
	1:30,000	+	+	+	+	+	+		
	1:60,000	+	+	+	+	+	+		
	1:120,000	+	+	+	+	+	+		
Control	.....	+	+	+	+	+	+		

— = sterile
+ = growth of pneumococcus

instead of 0.1 c.c. of culture was used, added to 5 c.c. of the solution of chemical.

Temperature.—Tests were conducted at both the standard temperature of 20 C. and at 37 C. in a specially constructed water bath adapted to the purposes of this work. The latter appeared to yield more regular results and higher bactericidal values.

Dilutions.—Dilutions of both the chemical under study and of the control phenol were prepared in the broth mentioned, instead of distilled water. The presence or absence of the few drops of blood did not appear to influence the results materially.

A carefully standardized 5% solution of phenol in distilled water was employed in all tests as a stock dilution, and to equalize matters 5% and 1% stock solutions of the various chemicals in sterile distilled water were first prepared and further dilutions made with the broth.

Seeding.—We have employed both the regulation 4-mm. loop of the Hygienic Laboratory method and graduated pipets (transferring 0.05 c.c.) for purposes

of inoculating the seed tubes. With the pneumococcus, the latter technic yielded more constant results by reason of transferring large numbers of cocci; but it has the disadvantages of transferring a portion of the chemical, and of being more difficult of execution.

Controls.—In each experiment several controls were included by placing 1 c.c. of culture in 5 c.c. of broth at 37 C. and subculturing with sterile pipets at the regular intervals of 2½, 5, 7½, 10, 12½, and 15 minutes.

Incubation.—All subcultures were incubated for 48 hours at 37 C., and then the results read and recorded.

Method of Conducting the Test and Determining the Coefficient.—With the modifications briefly described, the technic was conducted after the standard methods, and the coefficient determined, when possible, after the method of the Lancet Commission, which was adopted into the Hygienic Laboratory method, namely, the mean between the strength and time coefficients .

A fairly typical experiment selected from a number conducted with these modifications is shown in Table 1.

The results, as shown in Table 1, were irregular and generally even poorer than the illustration, despite a considerable number of repetitions by two different workers. The straight Rideal-Walker and Hygienic Laboratory methods were entirely unsatisfactory in work with the pneumococcus and the modified method described, while yielding better results, was found inferior to other methods to be detailed later. With B. typhosus as the test organism, however, these methods have proved of value in our experience in testing the bactericidal activity of new compounds, during the course of other studies in bacterial chemotherapy. A further modification consisting of plating instead of seeding the broth tubes shows when there is partial killing and diminution in the numbers of bacilli.

CENTRIFUGE METHOD

Bactericidal tests were also conducted with what may be called a centrifuge method, in which the bactericide is permitted to act on the pneumococci for a certain length of time, after which the microorganisms are removed for culture.

One cubic centimeter of varying dilutions of the test substance in sterile broth, was placed in each of a series of sterile test tubes suitable for centrifugation at high speed and furnished with sterile rubber stoppers. To each of these tubes and to each of several controls (1 c.c. of broth alone) was carefully added 1 c.c. of a 24-hour broth culture of a pneumococcus, and the whole gently mixed and incubated for 1 hour. At the end of this time from 6 to 8 c.c. of sterile broth were added to each tube, and the contents centrifugated at high speed for from 15 to 20 minutes. The supernatant fluid was then removed under sterile conditions, 6 to 8 c.c. of sterile broth added, and after the mass of cocci had been stirred up with a platinum wire, the

whole was centrifugated for a similar period in order to remove all traces of the chemical. After the washing, fresh sterile broth was added to each tube and this culture incubated for 48 hours, after which the results were read.

The results of experiments with 3 different quinin compounds are shown in Table 2.

TABLE 2

RESULTS OF BACTERICIDAL TESTS BY THE CENTRIFUGE METHOD WITH PNEUMOCOCCUS TYPE I (EXPOSURE OF 1 HOUR)

Test	24-hr. Broth Culture	Final Dilution	Results		
			Quinin and Urea Hydrochlorid	Ethylhydrocuprein Hydrochlorid	Hydroquinin Hydrochlorid
1	1 c.c.	1:100	—	—	—
2		1:200	+	—	+
3		1:300	+	—	+
4		1:400	+	—	+
5		1:500	+	—	+
6		1:600	+	—	+
7		1:700	+	—	+
8		1:800	+	—	+
9		1:900	+	+	+
10		1:1000	+	+	+
11		Control	+	+	+

— = sterile
+ = growth

This method consumes considerable time and requires extreme care to prevent contamination, but its results, as a rule, are sharp and definite. By varying the interval of exposure, information may be gained as to the rapidity of bactericidal action.

As shown in the table, ethylhydrocuprein hydrochlorid was 8 times more bactericidal than quinin and urea hydrochlorid and hydroquinin hydrochlorid.

PIPET METHOD

For comparison of the bactericidal activity of quinin compounds in solutions of sera and in normal salt solution, we have found the following method useful, because of the small amounts of serum required for a large number of tests.

Both ordinary capillary and looped pipets (Wright) prepared of ordinary 7-mm. glass tubing were used. The mouth end of each was plugged with cotton and all sterilized by dry heat before use.

Varying dilutions of the compound were prepared in sterile normal human or horse serum in quantities of 1 c.c., in a series of small sterile tubules held in a slanting position in plasticine and inoculated with 0.2 c.c. of a 24-hour broth culture of a pneumococcus. (These sera alone were without appreciable bactericidal effect on the pneumococci.)

At intervals of 5, 10, 15, 20, and 30 minutes, each dilution was subcultured by drawing a small portion of each, averaging about 5 c.mm., into the

pipet, the bulb of which had been filled just before with a special pneumococcus blood dextrose broth.

Each pipet was sealed in a peep flame immediately after filling and incubated for 48 hours, after which the results were read.

In each experiment a set of controls, prepared by adding 0.2 c.c. of culture to 1 c.c. of serum or salt solution respectively and subculturing at the same intervals, was included.

Tables 3, 4, and 5 show the results observed with this method with dilutions of ethylhydrocuprein hydrochlorid in sterile human serum acting on stock cultures of pneumococci belonging to Types I, II, and III.

The results obtained with this method were clear cut and sharp, and it possesses the distinct advantage of affording a practical and economical means of determining the bactericidal activity of substances in a menstruum of either serum, ascites fluid, salt solution, or distilled water.

With this method we have tested the bactericidal activity of quinin compounds and other disinfectants in solution in serum and salt solution on pneumococci of various types, the results being summarized elsewhere.[9]

THE COMBINED IN-VITRO-VIVO METHOD

In this method 1 c.c. of a dilution of the bactericide in serum, broth, or salt solution is allowed to act on 1 c.c. of a culture of pneumococci of known virulence so diluted that 0.1 c.c. contains 100 minimal lethal doses, at a temperature of 37 C. and at intervals of 5, 15, 30, 45, and 60 minutes; 0.2 c.c. of the mixture is injected intraperitoneally into mice to determine when killing of the pneumococci has occurred.

The technic of this test is as follows:

1. A 24-hour broth culture of a pneumococcus is titrated by intraperitoneal injection into mice to determine the M. L. D. in a period of 24-36 hours after injection. This culture is then diluted with sterile broth until 100 times the M. L. D. is contained in each 0.1 c.c.

2. A dilution of the bactericide in serum, broth, or salt solution is prepared —for example, 1:1000 ethylhydrocuprein hydrochlorid—and 1 c.c. placed in a small sterile tubule in a water bath at 37 C. After a few minutes 1 c.c. of the diluted culture is added and the contents gently mixed. The dilution of bactericide has now been doubled (1:2000).

3. At intervals of 5, 15, 30, 45, and 60 minutes, 0.2 c.c. of the mixture is injected into the peritoneal cavity of a white mouse. The 1-c.c. graduated syringe used for this purpose is sterilized before each injection.

4. One or more controls are included by mixing 1 c.c. of the diluted culture with 1 c.c. of the same fluid used in preparing the dilution of disinfectant (serum, broth, or salt solution); the tubule or tubules are kept in the water bath and 0.2 c.c. injected into a series of mice at the same intervals.

TABLE 3

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYLHYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE I)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	—	—	—	—	—
5	1:100,000	+	—	—	—	—
6	1:200,000	+	+	+	+	+
7	1:500,000	+	+	—	+	+
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

TABLE 4

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYLHYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE II)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	+	+	+	+	+
5	1:100,000	+	+	+	+	+
6	1:200,000	+	+	+	+	+
7	1:500,000	+	+	+	+	+
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

TABLE 5

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYLHYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE III)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	—	—	—	—	—
5	1:100,000	+	+	—	—	—
6	1:200,000	+	+	+	+	+
7	1:500,000	+	+	+	+	+
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

5. The mice are kept under observation for at least 6 days, and at autopsy the blood of the heart is examined by smear and culture for pneumococci. During the first 48 hours death is generally due to pneumococci; after this time the blood is usually found sterile and death is ascribed to the toxicity of the drug injected.

The results of a test conducted in this manner are shown in Table 6.

TABLE 6

RESULTS OF BACTERICIDAL TESTS BY THE COMBINED IN-VITRO-VIVO METHOD

Culture: 24-hour broth culture of pneumococcus, Type I; 0.00001 c.c. fatal by intraperitoneal injection in 48 hours; diluted with broth so that 0.1 c.c. contained 100 M.L.D.
Substance: quinin bisulfate; dilution 1:100 in sterile salt solution.
Proportion of bactericide and culture: 1 c.c. + 1 c.c. diluted culture (final dilution of bactericide then acting on the cocci was 1:200).

Test	Exposure (min.)	Result (days)						Remarks
		1	2	3	4	5	6	
1	5		Died					Pneumococci in blood
2	15						Living	
3	15						Living	
3	30						Living	
4	45						Living	
5	60						Living	
6	Control	Died						

The results observed with this method in testing the bactericidal activity of a number of quinin compounds and derivatives were comparatively definite. With different cultures of the same serologic type of pneumococcus, however, slightly varying results were observed, so that as usual in such animal experiments it is advisable to conduct an experiment in duplicate in order that true results may be obtained.

The method has the distinct advantage of controlling the result by animal inoculation, which in the case of a highly virulent culture of a pneumococcus constitutes in our experience a delicate test for viable microorganisms. The disadvantage of the test lies in the fact that in such a combined in-vitro-vivo method, some of the drug is injected intraperitoneally and probably continues its bactericidal activity within the peritoneal cavity for a short time at least. For example, in the experiment detailed, 0.001 gm. of quinin bisulfate was injected into each of the mice (averaging about 15 gm. in weight), corresponding to about 0.06 gm. per kilogram of body weight.

ANTISEPTIC METHOD

This method, which is an adaptation of older methods, is called an 'antiseptic' test, because it consists in using high dilutions of the disinfectant against small numbers of the microorganism over a long period of time.

This method has proved in our hands one of the best because of the sharpness of its results, the ease of its manipulation, and its adaptability for testing substances which are practically insoluble in water, salt solution, or broth.[10]

It is conducted as follows:

1. In a series of 10 sterile test tubes are prepared dilutions of the disinfectant in 1 c.c. of sterile broth or normal salt solution, which are 10 times higher than the final dilutions desired.

2. To each tube are added 9 c.c. of sterile broth (in pneumococcus work it is not necessary to use blood in the broth); to the control tube are added 1 c.c. of the same fluid used in preparing the dilutions of disinfectant and 9 c.c. of broth.

3. All tubes are now sterilized in the Arnold sterilizer for one-half hour and incubated over night to test for sterility of the disinfectant and broth and to bring the temperature to 37 C. (In the case of quinin and mercurial compounds the disinfectant values are not impaired by the process of sterilization; with volatile disinfectants it is better practice to omit sterilization, but not the preliminary incubation.)

4. On the following day each tube is seeded with a drop of pneumococcus culture (about 0.05 c.c.) and gently mixed. This does not materially alter the dilution.

5. All tubes are incubated at 37 C. for at least 8 days. As growths appear in the tubes, these are tested for purity and to guard against accidental contamination, by means of stained smears.

6. The results are read and recorded by daily inspection of the tubes. The controls invariably show a good growth after 24 hours' incubation. Perfectly clear tubes are recorded as sterile. On successive days lower and lower dilutions may show a growth of pneumococci up to the 6th day, after which the results remain stationary over a long period of time. The highest values of the bactericide are found on the first day; after this time inhibition may be overcome in one or more of the dilutions and growths occur. In the case of B. typhosus and staphylococci, growths may appear as late as the 7th day, but very seldom thereafter. We have recorded the bactericidal value of a substance according to the highest dilution remaining sterile on the 8th day.

In so far as the pneumococcus is concerned, tests of the clear or sterile tubes after 8 days by subculture in blood agar and by intraperitoneal injection into mice and intravenous injection into rabbits, are practically always negative, thus indicating that the microorganisms have been 'completely killed.'

The results of a few experiments with pneumococci of various types and various quinin derivatives are shown in Tables 7 to 12.

The results observed with a large number of substances as tested by this method are given elsewhere;[9] here it may be stated that on account of the uniformity of results as determined by different workers using the technic with the same set of substances, the sharpness of the results, the ease of manipulation and possibility of detecting finer

[10] Schamberg and Kolmer, Jour. Am. Med. Assn., 1914, 62, p. 1950.

TABLE 7

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	+	+	+	+	+	+
14			1:3,000,000	—	—	+	+	+	+	+	+
15			1:4,000,000	—	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 8

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN HYDROCHLORID AND PNEUMOCOCCUS TYPE II

Test	Culture	Broth	Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	—	—	—	—	—	—
14			1:3,000,000	—	—	—	—	—	—	—	—
15			1:4,000,000	—	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 9

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN HYDROCHLORID AND PNEUMOCOCCUS TYPE III

Test	Culture	Broth	Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	+	+	+	+	+	+
14			1:3,000,000	—	+	+	+	+	+	+	+
15			1:4,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 10

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING QUININ BISULFATE AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	+	+
9			1:200,000	+	+	+	+	+	+	+	+
10			1:800,000	+	+	+	+	+	+	+	+
11			1:1,000,000	+	+	+	+	+	+	+	+
12			1:1,500,000	+	+	+	+	+	+	+	+
13			1:2,000,000	+	+	+	+	+	+	+	+
14			1:3,000,000	+	+	+	+	+	+	+	+
15			1:4,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 11

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING QUININ AND UREA HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:1000	—	—	—	—	—	—	—	—
2			1:2500	—	—	—	—	—	—	—	—
3			1:5000	—	—	—	—	—	—	—	—
4			1:10,000	—	—	—	—	—	—	—	—
5			1:50,000	—	+	+	+	+	+	+	+
6			1:100,000	+	+	+	+	+	+	+	+
7			1:250,000	+	+	+	+	+	+	+	+
8			1:500,000	+	+	+	+	+	+	+	+
9			1:750,000	+	+	+	+	+	+	+	+
10			1:1,000,000	+	+	+	+	+	+	+	+
11			1:2,000,000	+	+	+	+	+	+	+	+
12			1:3,000,000	+	+	+	+	+	+	+	+
13			1:4,000,000	+	+	+	+	+	+	+	+
14			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 12

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING HYDROQUININ HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:15,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:30,000	—	—	—	—	—	—	—	—
7			1:40,000	—	—	—	—	—	—	—	—
8			1:50,000	—	—	—	—	—	—	—	—
9			1:80,000	—	+	+	+	+	+	+	+
10			1:100,000	—	+	+	+	+	+	+	+
11			1:200,000	+	+	+	+	+	+	+	+
12			1:800,000	+	+	+	+	+	+	+	+
13			1:1,000,000	+	+	+	+	+	+	+	+
14			1:2,000,000	+	+	+	+	+	+	+	+
15			1:3,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

grades and degrees of antiseptic and bactericidal power, we can recommend the method as a simple one for testing new compounds both soluble and insoluble, particularly the latter, in the course of chemotherapeutic or other studies.

PLATING METHOD

This bactericidal test endeavors to determine the dilution of drug producing death of some considerable part or all of the test microorganisms (partial or total germicidal action) in a given period of time, by removing a very small amount of the mixture of disinfectant and culture, and plating with sufficient medium to practically dilute out of action the small quantity of disinfectant carried over.

TABLE 13

RESULTS OF BACTERICIDAL TESTS BY THE PLATE METHOD

Substance: ethylhydrocuprein hydrochlorid.
Culture: 24-hour broth culture of pneumococcus, Type I.

Test	Dilutions	Results in 48 Hours	
		Plates	Test Tubes
1	1 : 10,000	Sterile	Sterile
2	1 : 20,000	Sterile	Sterile
3	1 : 40,000	Sterile	Sterile
4	1 : 80,000	Inhibition	Growth
5	1 : 100,000	Inhibition	Growth
6	1 : 200,000	Inhibition	Growth
7	1 : 400,000	Inhibition	Growth
8	1 : 1,000,000	Inhibition	Growth
9	1 : 2,000,000	Growth	Growth
10	1 : 4,000,000	Growth	Growth
11	Control	Growth	Growth

The test is conducted as follows:

1. A series of dilutions of the disinfectant in serum, ascites fluid, broth, or normal salt solution in amounts of 1 c.c. is prepared in sterile test tubes of sufficient size to hold 10 c.c. A stock dilution of the disinfectant in distilled water or salt solution is prepared and sterilized beforehand.

2. These tubes are incubated at 37 C. for 24 hours to determine sterility and to bring the temperature to 37 C.

3. To each tube is now added 1 c.c. of a 1:10 dilution of a 24-hour broth culture of the test microorganism; the final dilution of disinfectant acting on the microorganisms is thereby doubled.

4. Controls are prepared in the same manner, the disinfectant being omitted.

5. All tubes are now incubated for 24 hours; then 0.05 c.c. or a 4-mm. loopful, of each dilution and control is transferred to sterile petri dishes and cultured with from 8 to 10 c.c. of blood agar-agar (at 40 C.).

6. To the test tubes are now added 8 c.c. of broth.

7. Plates and test tubes are incubated 48 hours, and thereafter examined and the results recorded.

8. The test-tube cultures act as checks on the plates in determining the dilution in which there is complete killing.

The results of an experiment with this technic are shown in Table 13.

In this particular experiment a dilution of 1:40,000 proved bactericidal and one of 1:1,000,000 proved slightly antiseptic; cultivating the plates and test tubes for a longer time shows a variation in results, particularly with reference to the inhibitory, or antiseptic, dose.

With blood agar as the plating medium, we have not found it possible to make accurate counts of the plates; likewise the sediment which may be present in ascites fluid obscures colonies and renders counts inaccurate. All tests have been done in duplicate or in triplicate and the results recorded as they compared with the controls.

SUMMARY

In-vitro bactericidal tests are probably of value in chemotherapeutic studies as based on the general observation that substances most parasiticidal in vitro also show this quality in marked degree in vivo.

In-vitro tests are of value especially for preliminary trials and orientation and as a delicate and trustworthy means of detecting increasing parasitotropism in building up a monotropic chemical.

In-vitro tests should be conducted when possible with the microparasite causing the malady under study, as the object of chemotherapy should be the production not only of polybacteriotropic and polyprotozootropic chemicals, but also of monotropic substances for a definite microparasite. In the absence of a pure culture of the particular microparasite under study, a closely allied species may be used.

In conducting in-vitro tests in chemotherapeutic studies 2 main methods may be used: (1) a simple technic fulfilling the primary laws governing these chemical reactions, and (2) a test embracing as many of the factors believed to be operative in vivo, as possible.

It is highly important to adopt a definite technic and adhere to it in every detail in conducting these tests, because different methods yield varying results, depending mainly on whether the substance excels as a bactericide (killing quickly in high concentration, but losing rapidly in bactericidal power in low concentration) or as an antiseptic (retaining bactericidal power to a better extent in low concentration).

As a routine test characterized by simplicity and sharpness of results we have found the antiseptic test, or tube method, as described, to be of particular value. For detecting degrees of bactericidal and antiseptic activity, the plate method is quite satisfactory. For conducting tests in a menstruum of serum, the pipet method and the combined in-vitro-vivo method are to be recommended.